Molecular Immunology 46 (2009) 2218–2225

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

# Chicken scavenger receptors and their ligand-induced cellular immune responses

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ARTICLE INFO

*Article history:*
Received 23 February 2009
Received in revised form 7 April 2009
Accepted 16 April 2009
Available online 14 May 2009

*Keywords:*
Scavenger receptor
Nitric oxide
Oxidative burst
Innate immune response
Heterophil
Macrophage
Chicken

ABSTRACT

The scavenger receptors (SRs) comprise structurally and functionally divergent groups of cell surface and secreted proteins that play an important role in innate immune defenses. Searching translated chicken genomic databases revealed many proteins homologous to mammalian SRs. SR mediated immune functions (oxidative burst, degranulation, phagocytosis, nitric oxide (NO) production, and cytokine expression) were evaluated in chicken heterophils, peripheral blood mononuclear cells (PBMC), and a chicken macrophage cell line (HD11) using various SR class A and B ligands. Results showed that the SR-A ligands, fucoidan, poly(I) and poly(G), but not SR-B ligands, phosphatidylserine and LDL, stimulated dose-dependent NO production in HD11 cells. However, SR-A ligands failed to induce NO in chicken monocytes. Quantitative RT-PCR indicated that SR ligands differentially regulated the gene expression of cytokines and chemokine in HD11 cells with a strong up-regulation of the cytokines IL-1β and IL-6 and the chemokine MIP-1β, but had no effect on IL-4, IL-12, IFN-γ, and IFN-β. SR-B ligands did not alter expression of these genes. SR-A ligands had no stimulatory effect on functional response in heterophils. However, LDL, a SR-B ligand stimulated oxidative burst in both heterophils and PBMC. Additionally, results indicate that SRs are involved in bacterial binding in macrophages.

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## 1. Introduction

The SR superfamily encompasses eight different subclasses (A–H) of structurally diverse, but evolutionarily conserved cell surface or secreted proteins (Plüddemann et al., 2007). In mammals, myeloid cells (macrophages and dendritic cells) and selected endothelial cells are the primary cell types that express these scavenger receptor cysteine rich (SRCR) domain-containing proteins (Plüddemann et al., 2007). One of the prominent characteristics of SRs is their ability to bind a range of extremely diverse ligands, including both Gram-positive and Gram-negative bacteria, a variety of polyanions including the bacterial cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA), DNA and RNA oligonucleotides, environmental particles, endogenous and modified host-derived molecules, and apoptotic cells (Peiser et al., 2002b; Plüddemann et al., 2007). SRs' binding affinity to microbial molecules and their preferential expression on cells of innate immune function strongly suggests an important role of SRs in the host immune defense against pathogens. Thus far, SRs have been shown to participate in the resolution of bacterial infections by mediating antimicrobial activity, facilitating phagocytosis of bacteria, and clearing bacterium-derived endotoxins from circulation (Hampton et al., 1991; Dunne et al., 1994; van der Laan et al., 1999; Thomas et al., 2000; Peiser et al., 2002a; Mukhopadhyay et al., 2006; Amiel et al., 2007). Exposure of mammalian immune cells to SR ligands induces immune responses which include production of nitric oxide (NO) (Campa et al., 2005; Nakamura et al., 2006) and expression of proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-18, and IL-12 (Hsu et al., 2001; Mytar et al., 2004; Limmon et al., 2008; Villwock et al., 2008). Additionally, SRs mediate uptake of exogenous antigens and actively participate in cross-presentation of antigen in dendritic cells, indicating that SRs are involved in development of the adaptive immune response (Delneste, 2004; Shakushiro et al., 2004; Barth et al., 2008; Harvey et al., 2008).

SRs and their function in the chicken immune system are mostly unknown. However, an earlier study (Vandaveer et al., 2001) indicated involvement of SR in the differentiation of Th1 immune response in chickens. In the present study, we profiled chicken SRs through in silico analysis of chicken genomic/protein database and examined the immune responses of chicken peripheral blood heterophils, PBMC, monocytes, and a chicken macrophage cell line, HD11, to stimulation with various SR ligands. This is the first report to identify chicken SRs and their immune function in chicken immune cells.

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0161-5890/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.molimm.2009.04.020

## 2. Materials and methods

### 2.1. In silico search of chicken proteins homologous to human scavenger receptors

To identify putative SRs in the chicken, all known SR protein sequences (SR class A–H) and conserved SRCR domain sequences discovered in human, mouse, and *Drosophila* (SR class C) were individually queried against the translated chicken genomic BLAST databases in GenBank by using the Protein BLAST program with default settings on the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/). For all potential hits, the protein sequences were further analyzed for the presence of conserved SR domain structure and sequence similarity to the human or mouse equivalents.

### 2.2. Reagents

SR-A ligands: fucoidan, poly(G), and poly(I); SR-B ligands phosphatidylserine (Pds) and native LDL; non-SR ligands poly(A) and poly(C); and media and other reagents used for cell isolation and culture were purchased from Sigma (St. Louis, MO, USA).

### 2.3. Cell isolation

Chicken peripheral blood heterophils and mononuclear cells (PBMC) were isolated from peripheral blood collected from 2- or 3-day-old chickens as previously described (He et al., 2005). Briefly, peripheral blood samples were mixed with 1% methylcellulose (1:1, v/v) and centrifuged at 25 × *g* for 15 min to remove red blood cells. The supernatants were then carefully layered onto a discontinuous gradient of Histopaque®-1077/1119 (10 ml each) in 50-ml conical centrifuge tubes, and centrifuged at 250 × *g* for 60 min. The PBMC at the 1.077/supernatant interface and heterophils in the Histopaque®-1119 phase were removed to new tube, collected by centrifugation, and resuspended in RPMI-1640.

### 2.4. Monocyte culture and stimulation

Monocytes were isolated from PBMC using plastic adhesion (He et al., 2007). Briefly, aliquots of 200 μl of PBMC ($1 \times 10^7$ cells/ml) containing 50 μg gentamicin/ml were dispensed to a round-bottomed 96-well cell culture plate and incubated at room temperature for 3 h. Non-adherent cells were removed by washing twice with RPMI-1640 and the adherent monocytes were cultured over night (to obtain quiescent state) in Dulbecco's modified Eagles medium (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml), and 1.5 mM L-glutamine at 41 °C in a 5% $CO_2$ and 95% humidity incubator. Prior to stimulation, the media was replaced with fresh culture medium to remove any additional detached cells, and cells were then stimulated with various agonists for 72 h.

### 2.5. Chicken macrophage cell line HD11 culture and stimulation

The HD11 cells were maintained in complete DMEM medium described as above. For NO production assays, 100-μl aliquots of $2 \times 10^6$ HD11 cell/ml were seeded into each well of 96-well round-bottom plates and incubated overnight (16–18 h). Media was replaced prior to stimulation and cells were stimulated with various SR ligands for 24 h in a final volume of 200 μl/well. For quantitative realtime PCR (QRT-PCR) analysis of cytokine gene expression, HD11 cells were seeded at $2 \times 10^6$ cell/well in 12-well plates and incubated overnight. The cells were then stimulated for 2 h with various SR ligands and harvested for total RNA isolation.

### 2.6. Nitrite assay

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Griess assay (Green et al., 1982). Briefly, an aliquot of 100 μl culture supernatant from each well was transferred to the wells of a new 96-well flat-bottom plate and combined with 50 μl of 1% sulfanilamide and 50 μl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution). After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density ($OD_{550}$) of each well using a SPECTRA MAX microplate reader (Molecular Devices, Sunnyvale, CA). Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in the cell-free medium.

### 2.7. Oxidative burst assay

Production of reactive oxygen species (ROS) by chicken heterophils and peripheral blood mononuclear cells during oxidative burst was measured by oxidation of 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) to fluorescent DCF as described (He et al., 2005). Briefly, chicken heterophils and mononuclear cells (1 ml of $1 \times 10^7$ cells/ml in RPMI) were stimulated with various SR ligands in 2-ml microcentrifuge tubes containing 10 μg/ml of DCFH-DA for 1 h at 37 °C in 5% $CO_2$ and 95% humidity. Cell culture aliquots (150 μl) were then dispensed to a black 96-well plate and the relative fluorescent units (RFU) were measured (485/530 nm) using a fluorescence microplate reader (Genios Plus Plate Reader, TECAN U.S. Inc., NC, USA). Phorbolmyristate acetate (PMA) stimulated cell culture was used as the positive control.

### 2.8. Degranulation assay

Heterophil degranulation was measured by quantifying β-glucuronidase activity (He et al., 2005) in culture medium following stimulation of heterophils ($8 \times 10^6$ ml$^{-1}$) with SR ligands at 41 °C for 60 min on a rocker platform in 5% $CO_2$ and 95% humidity. After incubation, the cells were pelleted by centrifugation at 10,000 × *g* for 2 min at 4 °C and supernatants were collected for the assay. Aliquot of 25 μl supernatant was incubated with 50 μl of freshly prepared substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide and 0.1% Triton X-100 in 0.1 M sodium acetate buffer) in a black 96-well plate for 4 h at 41 °C. The reaction was stopped by adding 200 μl of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone was measured fluorometrically (355/460 nm) using a fluorescence microplate reader.

### 2.9. QRT-PCR analysis of cytokine gene expression

Total RNA from HD11 cells was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA samples (1 μg each reaction) were DNase treated and reverse transcribed to cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen). Expressions of chicken cytokines (Table 1) and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were determined by QRT-PCR using the MX3000P® (Stratagene, La Jolla, CA). Primers and probes (Table 1) were obtained from Applied Biosystem (Austin, TX). Primer amplification efficiency was verified for each gene using 2-fold serial dilutions of cDNA. QRT-PCR was performed for each sample in duplicate in a total volume of 25 μl, consisting of 12.5 μl Brilliant® II QPCR Master Mix (Stratagene), 0.5 μl ROX reference dye diluted 1:500, 1.25 μl primer/probe mix (900 nM/250 nM final concentrations, respectively), 5.75 μl RNase/DNase-free water, and 5 μl diluted cDNA (25 ng RNA). All reaction plates were run under identical cycle conditions, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. The florescence threshold was

**Table 1**
QRT-PCR primer and probe sequences.

| Name | Sequence (5′ → 3′) | |
|---|---|---|
| GAPDH | Probe | CTTGGCTGGTTTCTCC-(FAM) |
| | F | CCCCAATGTCTCTGTTGTTGAC |
| | R | CAGCCTTCACTACCCTCTTGAT |
| IL-1β[a] | Probe | CCA CAC TGC AGC TGG AGG AAG CC-(FAM) |
| | F | GCT CTA CAT GTC GTG TGT GAT GAG |
| | R | TGT CGA TGT CCC GCA TGA |
| IL-4 | Probe | ACGCAGGAAACCTCTC-(FAM) |
| | F | AGCATCCGGATAGTGAATGACATC |
| | R | TCTGCAAAGATATCTGTCACGTTCA |
| IL-6 | Probe | CTTGCACATCTCGTCCTGC-(FAM) |
| | F | ACCGCGCCGTCCAG |
| | R | TTCTCGCACACGGTGAACTT |
| IL-12p40[a] | Probe | CTG AAA AGC TAT AAA GAG CCA AGC AAG ACG TTC T-(FAM) |
| | F | TGG GCA AAT GAT ACG GTC AA |
| | R | CAG AGT AGT TCT TTG CCT CAC ATT TT |
| IFN-β[a] | Probe | TTA GCA GCC CAC ACA CTC CAA AAC ACT G-(FAM) |
| | F | CCT CCA ACA CCT CTT CAA CAT G |
| | R | TGG CGT GTG CGG TCA AT |
| IFN-γ[a] | Probe | TGG CCA AGC TCC CGA TGA ACG A-(FAM) |
| | F | GTG AAG AAG GTG AAA GAT ATC ATG GA |
| | R | GCT TTG CGC TGG ATT CTC A |
| MIP-1β | Probe | ACCGGTGGGTTCTGAC-(FAM) |
| | F | CCTCATTGCCATCTGCTACCA |
| | R | CGGGAGATGTAGGTGAAGCA |

F: forward; R: reverse.
[a] Primer and probe sequences are kindly provided by Dr. Peter Kaiser, Institute for Animal Health, Compton, UK.

set at 0.2 and the resulting cycle threshold values (Ct), normalized to the reference gene, were used for analysis.

### 2.10. Evaluation of bacterial binding activity of macrophage SRs

HD11 cells were seeded at $2 \times 10^6$ cells/well in a 96-well plate and cultured overnight. To make horseradish peroxidase (HRP)-labeled *Salmonella enteritidis*, a primary poultry isolate *S. enteritidis*, obtained from the National Veterinary Services Laboratory, Ames, IA, was used to prepare formalin-killed *S. enteritidis* (FKSE) by incubating the bacteria in a 0.5% formalin solution at 4 °C for 24 h. The FKSE was washed three times with PBS to remove the formalin and $1 \times 10^8$ bacteria was then conjugated with HRP using the SureLINK™ HRP Conjugation Kit (KLP, Gaithersburg, MD). The competitive binding assay was carried out by first incubating the cells with SR ligands for 1 h followed by incubating with HRP-*S. enteritidis* for 1 h, washing three times with PBST (0.1% Tween in PBS) to remove unbound HRP-*S. enteritidis*, and then incubating with HRP substrate, SIGMAFAST™ OPD (Sigma), for 30 min. The optical density ($OD_{450}$) was measured using a SPECTRA MAX microplate reader.

### 2.11. Data analysis

Values are presented as mean ± SD from at least three independent experiments. Statistically significant differences were determined at the level of $P < 0.05$ by the Student's *t*-test using the SigmaStat software (Jandel Corp, CA, USA).

## 3. Results

### 3.1. In silico profiling chicken scavenger receptors

Many annotated and unannotated chicken proteins homologous to SRs found in mammals were discovered by searching translated chicken genomic databases with Protein BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) using protein or peptide sequences of the conserved SRCR domain and individual SR found in human and mouse (Table 2). These putative chicken SRs include three members of SR-A: macrophage receptor with collagenous structure (MARCO), scavenge receptor class A member 5 (SCARA5), and scavenger receptor with C-type lectin (SRCL); three members of SR-B: SCARB1, SCARB2, and CD36/SCARB3; two members of SR-F: SCARF1 and partial sequence of SCARF2; and one SR-H: FELE-1. The database search, however, failed to identify homologous chicken sequences to human CD68 (SR-D), LOX-1 (SR-E), and R-PSOX (SR-G). Sequence alignment analysis (http://www.genebee.msu.su/genebee.html) demonstrates that these chicken putative SRs share high homology with their human equivalents (Table 2). Additionally, BLAST search using SRCR domain sequences identified (not included in the Table 2) many other SRCR domain-containing proteins which are structurally similar to mammalian CD5 (NP_990525 and XP_424435), CD6 (XP_424583), and CD163 (XP_416526, XP_421056, XP_421057, XP_421058, XP_423532, XP_001231760, XP_001231884, XP_001231940, XP_001233355, XP_001235597, XP_001235815, XP_001235941, XP_001236120, XP_001236123). Several sequences (XP_424429, XP_424430, XP_424433) were found to resemble the protein of Deleted in Malignant Brain Tumors 1 (DMBT-1), which has been shown to play important roles in tumor suppression and pathogen defense (Mollenhauer et al., 1997; Rosenstiel et al., 2007). These SRCR domain-containing proteins are referred to as SRs in the literature, but they have not been proven to bind modified LDL or other polyanionic ligands, which typically defines a SR and therefore do not belong to the classified SRs (Plüddemann et al., 2007).

**Table 2**
Chicken proteins homologous to human SRs.

| Chicken SRs | Class | Ref. seq. accession no. | Length (aa) | Identity (%)[a] | Conserved domains[b] |
|---|---|---|---|---|---|
| MARCO | A | NP_990067 | 476 | 48 | SRCR, collagen |
| SCARA5 | A | XP_001234366 | 495 | 73 | SRCR |
| SRCL | A | NP_001034688 | 742 | 83 | CLECT_DC-SIGN_like, Smc |
| SCARB1 | B | XP_415106 | 503 | 70 | CD36 |
| SCARB2 | B | XP_420593 | 481 | 70 | CD36 |
| CD36/SCARB3 | B | NP_001025902 | 471 | 72 | CD36 |
| SCARF1 | F | XP_001234823 | 778 | 59 | EGF-like repeats |
| SCARF2 | F | XP_001234051 | 285[c] | 72 | EGF-like repeats |
| FELE-1 | H | XP_414246 | 2209 | 55 | Fasciclin, EGF-like, C-type lectin-like hyaluronan-binding link module |

[a] Compared to human SR sequences.
[b] CLECT_DC-SIGN_like: C-type lectin-like domain. Smc: chromosome segregation ATPases. SRCR: scavenger receptor cysteine-rich domain. Collagen: collagen triple helix repeat. CD36: CD36 antigen family. EGF-like repeats: epidermal growth factor-like repeat domains.
[c] Partial sequence.

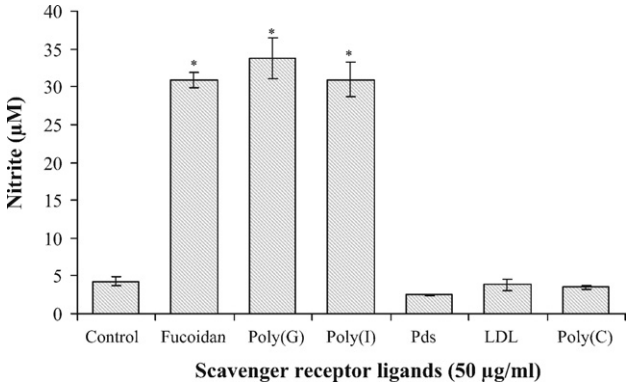


**Fig. 1.** Effect of SR ligands on NO production in chicken macrophage cell line HD11. Control: unstimulated cells; SR-A ligands: fucoidan, poly(G), and poly(C); SR-B ligands: Pds and LDL; non-SR ligand: poly(C). Data are means and standard deviation of three individual experiments with asterisk (*) indicating a significant difference ($P \leq 0.05$) in NO production between stimulated and unstimulated cells.

### 3.2. Induction of NO in HD11 cells by SR ligands

None of SR ligands tested in the present study induced NO production in chicken monocytes after 72 h incubation (data not shown). On the contrary, in chicken macrophage HD11 cells, a significant amount of NO was induced by SR-A ligands fucoidan, poly(G) and poly(I) after 24 h incubation. SR-B ligands Pds and LDL failed to stimulate NO production in HD11 cells (Fig. 1). Poly(C) as a non-SR ligand also did not stimulate NO production in HD11 cells. Inductions of NO in HD11 cells by fucoidan, poly(G), and poly(I) were does-dependent (Fig. 2) with 25 μg/ml of both poly(G) and poly(I) and more than 50 μg/ml of fucoidan required for the maximum NO inductions under the experimental conditions.

### 3.3. Effect of SR ligands on oxidative burst of peripheral blood heterophils and PBMC

Chicken heterophils are the avian equivalent to mammalian neutrophils. We examined oxidative burst response of chicken heterophils, and additionally peripheral blood mononuclear cells (PBMC), to stimulation with various SR ligands. Our results showed that most SR ligands did not induce oxidative burst, except for native LDL, which induced a strong oxidative burst response in both heterophils (Fig. 3) and PBMC (Fig. 4).

### 3.4. Effect of SR ligands on degranulation of heterophils

Degranulation is a host inflammatory response to bacterial infection, in which granulocytes such as heterophils release bactericidal substances. In this study, none of the tested SR ligands were found to stimulate degranulation of chicken heterophils (data not shown). It is interesting to note that although LDL stimulates strong oxidative burst, it failed to induce a degranulation response.

### 3.5. Induction of cytokine and chemokine expressions in HD11 cells by SR ligands

QRT-PCR was used to evaluate the effect of SR ligands on the expression of cytokines, including the proinflammatory cytokines IL-1β, IL6, and IFN-β, $Th_2$ cytokine IL-4, and $Th_1$ cytokines IL-12 and IFN-γ in HD11. In addition to cytokines, the expression of the chemokine, MIP-1β, was also examined (Fig. 5). After 2 h stimulation, SR-A ligands strongly up-regulated the expression of proinflammatory cytokines IL-1β and IL-6 and chemokine MIP-1β in HD11 cells, whereas IL-4, IL-12, IFN-γ, and IFN-β were not affected. Poly(C), a non-SR ligand, was unexpectedly found to also stimulate, to less extent, the expression of IL-1β and MIP-1β, but had no effect on IL-6 expression. SR-B ligands showed no effect on expression of any cytokines or chemokine.

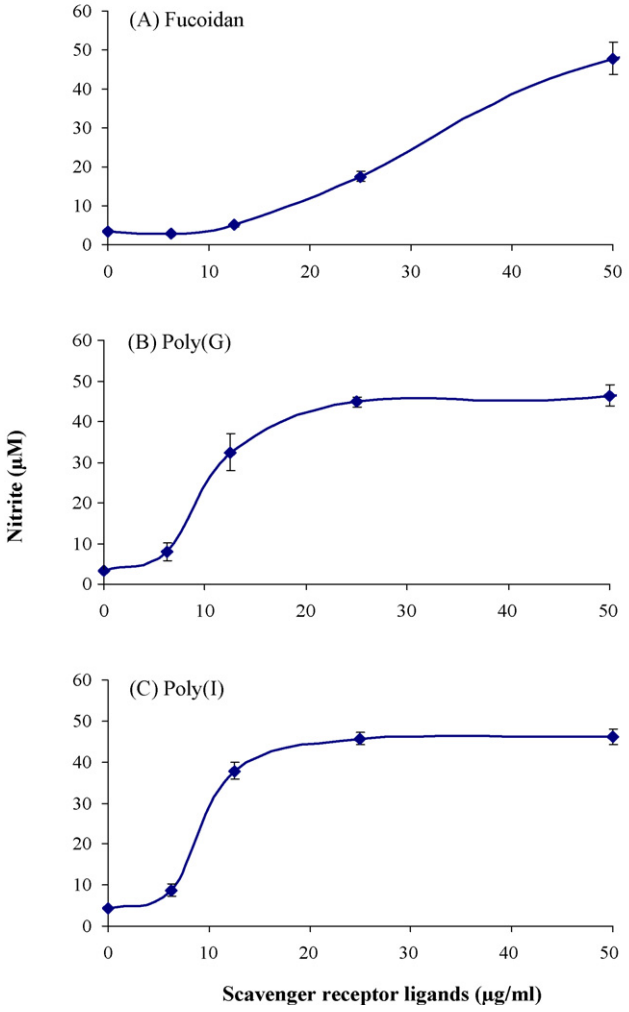


**Fig. 2.** Dose-dependent induction of NO in HD11 cells by SR-A ligands fucoidan, poly(G), and poly(I). Data are means and the standard deviation of three individual experiments.

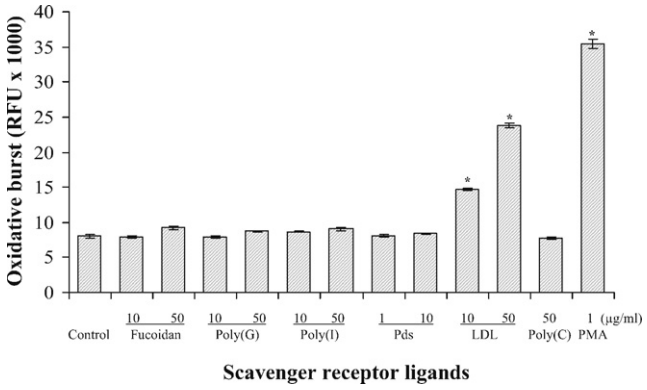


**Fig. 3.** Effect of SR ligands on oxidative burst of chicken heterophils. Control: unstimulated cells; SR-A ligands: fucoidan, poly(G), and poly(C); SR-B ligands: Pds and LDL; non-SR ligand: poly(C). PMA treated cell group was used as a positive control. Data are means and standard deviation of three individual experiments with asterisk (*) indicating a significant difference ($P \leq 0.05$) in oxidative burst between stimulated and unstimulated cells.

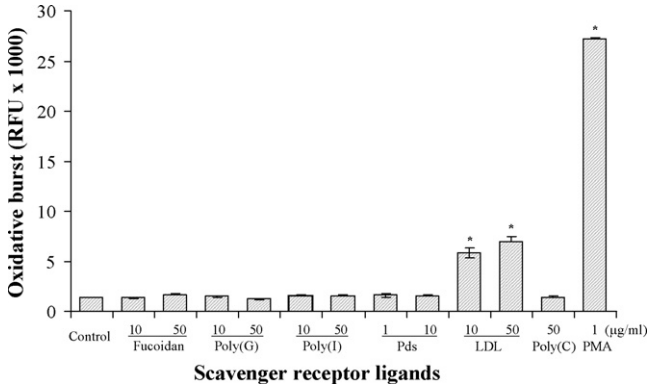


**Fig. 4.** Effect of SR ligands on oxidative burst of PBMC. Control: unstimulated cells; SR-A ligands: fucoidan, poly(G), and poly(C); SR-B ligands: Pds and LDL; non-SR ligand: poly(C). PMA treated cell group was used as a positive control. Data are means and standard deviation of three individual experiments with asterisk (*) indicating a significant difference ($P \leq 0.05$) in oxidative burst between stimulated and unstimulated cells.

### 3.6. Effect of SR ligands on macrophage binding of bacteria

SRs are known to engage in binding and uptake of bacteria by macrophage cells. This function of SRs was examined by ligand competition assay, in which the inhibitory effects of preincubation of HD11 cells with SR ligands on subsequent bacterial binding capacity of the cells were examined. Our results show that most SR ligands, except for Pds, competitively reduced the binding capacity of HD11 cells for HRP-*S. enteritidis* (Fig. 6). Poly(C) also reduced bacterial binding of HD11 cells. However, preincubation with another non-RS ligand, poly(A), did not reduce the binding activity of HD11 cells.

## 4. Discussion

In this study, with the available chicken genomic sequence database, we have identified and analyzed many putative protein sequences that share a high degree of homology to SRs found in mammals, including MARCO, SCARA5, SRCL, SCARB1, SCARB2, CD36/SCARB3, CD163, SCARF1, SCARF2, and FELE-1, belonging to SR class A, B, F, and H. The fact that these identified chicken SRs share high degree of homology with their mammalian counterparts indicates a high probability of functional conservation in chicken SRs. However, the database search failed to find sequences similar to mammalian SR class D (CD68E), E (LOX-1), and G (R-PSOX). Although the specific function of a particular chicken SR remains to be determined, profiling chicken putative SRs from the genomic database in the present study provides fundamental information that is useful for future study of chicken SRs and their function in the avian immune system.

Production of NO by activated monocytes/macrophages is an important innate immune response, playing a critical role in the host defense against both viral and bacterial infections (Bogdan, 2001; Chakravortty and Hensel, 2003). Previous studies (He et al., 2006a,b, 2007) have shown chicken primary monocytes produce large quantities of NO when exposed to bacteria, such as *Salmonella*, or when stimulated by PAMPs, particularly CpG oligodeoxydinucleotides which is the agonist for mammalian TLR9. In the present study, none of the SR-A ligands examined were able to induce NO production in chicken monocytes. However, it appears that maturation or differentiation of monocytes into macrophages is required for SR-A ligands to induce NO production, as the avian macrophage HD11 cells produce NO in response to AR-A ligand stimulation. The lack of NO production in immature primary monocytes in response to SR-A ligand stimulation is most likely due to the lack of expression of functional SR-A on the monocytes. SR-A receptors are only expressed on macrophages and dendritic cells, but not monocytes or neutrophils in mammalian species (Peiser et al., 2002b). Our result suggests the evolutionary conservation of the cell-type specific distribution of SRs among species. The chicken HD11 cell is a replication-defective avian leukemia virus MC29-transformed macrophage-like cell line with characteristics of normal macrophages (Beug et al., 1979). The HD11 cells have been shown to readily produce a significant amount of NO when stimulated with TLR agonists LPS, poly I:C, and CpG DNA (He and Kogut, 2003; He et al., 2007) and agonists that do not induce NO in monocytes, such as flagellin, poly I:C, and synthetic lipoprotein Pam3CSK4 (unpublished data). In the present study, HD11 cells showed differential responses to SR ligand stimulation, exhibiting dose-dependent NO production only responding to SR-A, but not to SR-B ligands. These results clearly indicate the expression of functional SR-A on HD11 cells and the involvement of these receptors in the innate immune function of chicken macrophages, which is in line with previous studies of mammalian macrophage cells (Campa et al., 2005; Nakamura et al., 2006). Although macrophages are known to express SR-B (Peiser et al., 2002b), ligands of SR-B have an inhibitory effect on iNOS, as shown in mouse peritoneal macrophages stimulated with LPS (Matsuno et al., 1997). On the other hand, the SR-B ligand HDL, but not LDL, mediates activation of eNOS in endothelial cells (Mineo and Shaul, 2003). SR-Bs, serving primarily as lipid scavengers with a high affinity for both native and oxidized HDL and LDL play a critical role in the lipoprotein and cholesterol metabolism and protection against coronary heart disease (Gu et al., 2000; Gillotte-Taylor et al., 2001; Eckhardt et al., 2004; Trigatti et al., 2004). These observations strongly suggest the possibility that induction of NO by SR-B ligands is both ligand-specific and cell-type dependent. In the present study, both SR-B ligands LDL and Pds have failed to stimulate NO in HD11 cells, indicating that unlike class-A SRs, class-B SRs may not mediate inflammatory immune response in the chicken macrophage cells.

In mammalian immune cells, exposure to ligands of SRs has been known to stimulate expression of proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-18, and IL-12 (Hsu et al., 2001; Mytar et al., 2004; Limmon et al., 2008; Villwock et al., 2008). Our results indicated differential effects of SR ligands on expression of these immune regulators in the chicken macrophage HD11. SR-A ligands induced a strong up-regulation of proinflammatory cytokine IL-1β and IL-6 and chemokine MIP-1β; whereas little effect was observed on the expression of IL-4, IL-12, IFN-γ, and IFN-β. Induced cytokine expression profile indicates that the class-A SRs promote primarily inflammatory immune response in chicken macrophages, which is in agreement with previous reports on the mammalian macrophages (Hsu et al., 2001; Mytar et al., 2004). The strong inflammatory immune response mediated by SR-A in chicken macrophages suggests SR-A has a role in the host immune response at the site of injury or infection. Similar to NO, SR-B ligands did not alter the expression of any of these genes. In contrast to human monocytes and macrophages where LDL stimulates increased expression of IL-12 (Mytar et al., 2004), this ligand has little effect on chicken macrophages. Poly(C), a non-SR ligand, has no effect on NO production and IL-6 expression, but was unexpectedly found to stimulate, though to a lesser degree, the expressions of IL-1β and MIP-1β. This suggests that, unlike mammalian SR-A, chicken SR-A may be able to loosely interact with poly(C).

SRs are known to play an important role in binding, uptake, and phagocytosis of bacteria by macrophages (Peiser et al., 2002b; Mukhopadhyay et al., 2006; Plüddemann et al., 2006). The bacterial binding activity of SRs in HD11 cells was evaluated by a 96-well microplate based ligand competitive binding colorimetric assay that measures the percentage inhibition of SR ligand pretreatments on the binding capacity of HD11 cells to horseradish peroxidase HRP-labeled *S. enteritidis*. Most SR ligands, except for

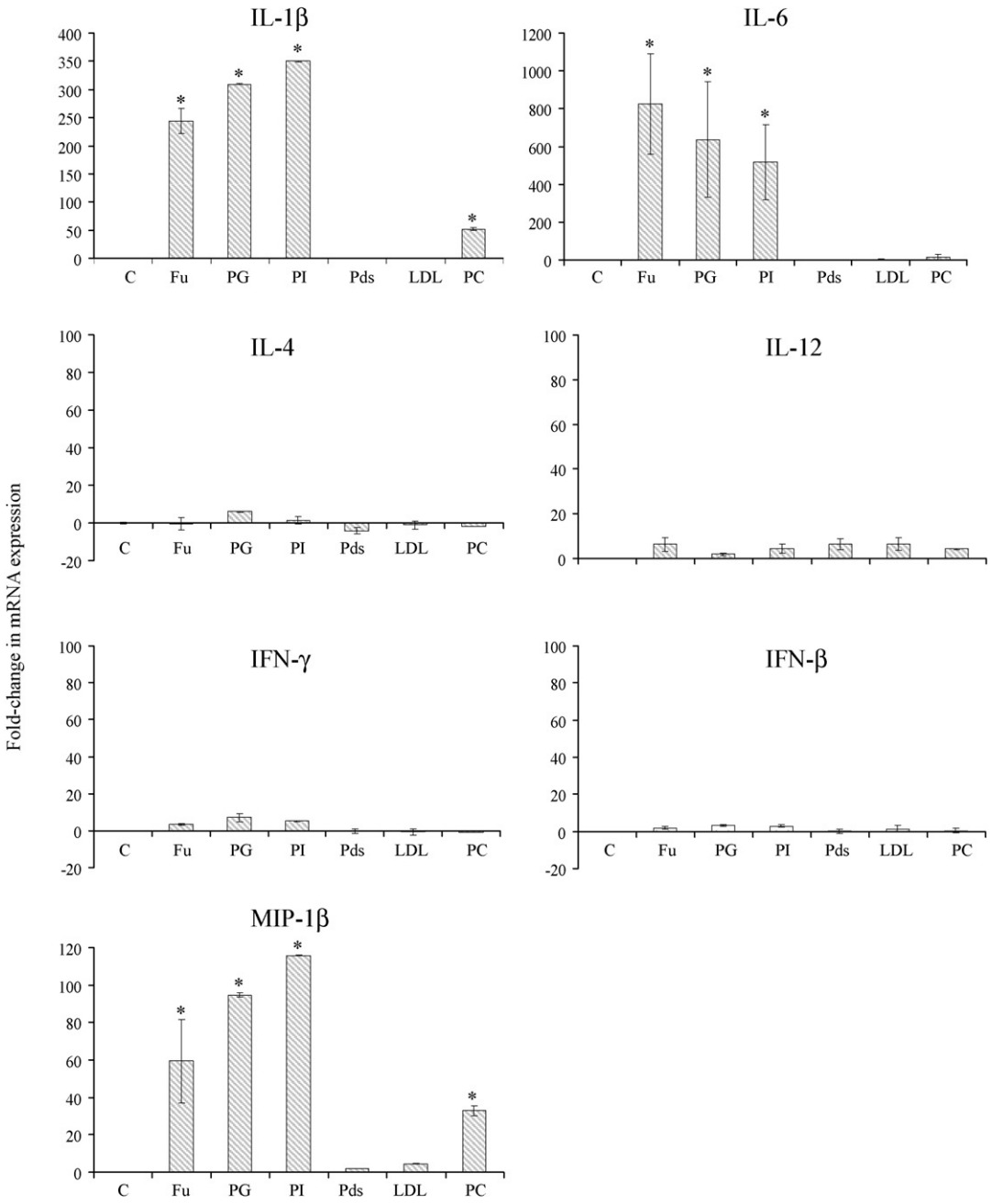


**Fig. 5.** Induction of cytokine and chemokine expressions in HD11 cells by SR ligands. Control: C; SR-A ligands: Fu (fucoidan), PG [poly(G)], and PI [poly(I)]; SR-B ligands: Pds and LDL; non-SR ligand: PC [poly(C)]. Data are means and standard deviation of two representative experiments with asterisk (*) indicating a significant difference ($P \leq 0.05$) in cytokine or chemokine expression (fold-change in mRNA levels) between stimulated and unstimulated cells.

Pds, competitively reduced the binding capacity of HD11 cells for HRP-*S. enteritidis*. While poly(C) also reduced bacterial binding of HD11 cells, preincubation with another non-RS ligand, poly(A), did not reduce the binding activity of HD11 cells. These results indicate that chicken SRs are involved in binding and phagocytosis of the Gram-negative *S. enteritidis*. The reduction in bacterial binding capacity after ligand-ligation of SRs confirms that SRs constitute an important part of receptor complex that are involved in the bacterial binding and uptake by macrophages.

Heterophil oxidative burst and degranulation activities are important bactericidal mechanisms of the cellular immune responses that play a critical role in the innate immune defense against invading pathogens in chickens (Kogut et al., 1995). Although SRs are restrictively expressed in myeloid cells (macrophages and dendritic cells) and certain endothelial cells (Peiser et al., 2002b; Plüddemann et al., 2006), we examined oxidative burst and degranulation activities to determine whether chicken heterophils respond to SR ligand stimulation. As anticipated, most SRs showed no stimulatory effect on heterophil oxidative burst and degranulation, with the exception of the SR-B ligand, LDL, which induced a strong oxidative burst reaction in both heterophils and PBMC. Human neutrophils and lymphocytes are known to express a LDL receptor which mediates LDL induce oxidative burst (Bonneau et al., 1994; Lara et al., 1997; De Sanctis et al., 1998). Although, the physiological role of the LDL receptor is to maintain homeostatic blood cholesterol by removing cholesterol-containing lipoprotein particles from circulation (Defesche, 2004), LDL receptor mediated oxidative burst in neutrophils implies the

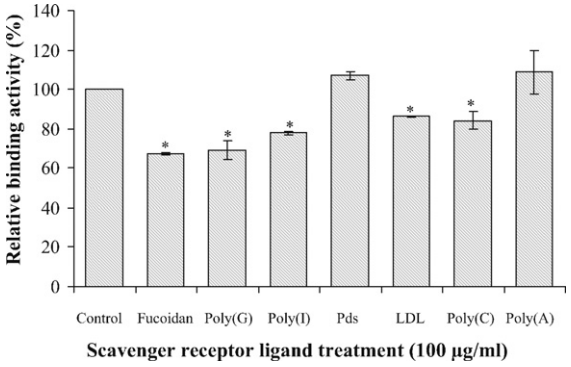


**Fig. 6.** Effect of SR ligand pretreatments on HD11 macrophage phagocytosis/binding of HRP-*S. enteritidis*. Control: untreated cells; SR-A ligands: fucoidan, poly(G), and poly(C); SR-B ligands: Pds and LDL; non-SR ligands: poly(C) and poly(A). Data are means and standard deviation of three individual experiments with asterisk (*) indicating a significant difference ($P \leq 0.05$) in the relative binding capacity (%) between ligand-treated and untreated (control) cells.

potential involvement of these cells in oxidation of LDL under the physiological condition and pathogenesis of vascular endothelial dysfunction (Holvoet, 1999). It is interesting to note that human-derived LDL induced a strong oxidative burst response in chicken heterophils. We believe that LDL induced oxidative burst in chicken heterophils is mediated by the LDL receptor, since heterophils are the chicken equivalent to mammalian neutrophils. However, the physiological significance of this finding requires future investigation.

In summary, our study has identified many annotated or unannotated chicken homologues to mammalian SRs through searching of the translated chicken genomic databases. Using SR ligands, we demonstrated that the A class SRs, but not the B class SRs, mediated strong proinflammatory immune responses in the chicken macrophage cell line HD11, including NO production and expression of cytokines IL-1β and IL-6 and chemokine MIP-1β. Additionally, both SR-A and SR-B were shown to be involve in the bacterial binding activity of chicken macrophage cells. We also found LDL to be a potent stimulant for chicken heterophil oxidative burst, which we believe is mediated by the LDL receptor.

## Acknowledgements

The authors thank Laura Ripley for her assistance in cell culture. Mention of commercial or proprietary products in this paper does not constitute an endorsement of these products by the USDA, nor does it imply the recommendation of products by the USDA to the exclusion of similar products.

## References

Amiel, E., Nicholson-Dykstra, S., Walters, J.J., Higgs, H., Berwin, B., 2007. Scavenger receptor-A functions in phagocytosis of *E. coli* by bone marrow dendritic cells. Exp. Cell. Res. 313, 1438–1448.

Barth, H., Schnober, E.K., Neumann-Haefelin, C., Thumann, C., Zeisel, M.B., Diepolder, H.M., Hu, Z., Liang, T.J., Blum, H.E., Thimme, R., Lambotin, M., Baumert, T.F., 2008. Scavenger receptor class B is required for hepatitis C virus uptake and cross-presentation by human dendritic cells. J. Virol. 82, 3466–3479.

Beug, H., von Kirchbach, A., Döderlein, G., Conscience, J.-F., Graf, T., 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18, 375–390.

Bogdan, C., 2001. Nitric oxide and the immune response. Nat. Immunol. 2, 907–916.

Bonneau, C., Couderc, R., Roch-Arveiller, M., Giroud, J.P., Raichvarg, D., 1994. Effects of low-density lipoproteins on polymorphonuclear leukocyte functions in vitro. J. Lipid Mediat. Cell Signal. 10, 203–212.

Campa, V.M., Iglesias, J.M., Carcedo, M.T., Rodríguez, R., Riera, J., Ramos, S., Lazo, P.S., 2005. Polyinosinic acid induces TNF and NO production as well as NF-kappaB and AP-1 transcriptional activation in the monocyte/macrophage cell line RAW 264.7. Inflamm. Res. 54, 328–337.

Chakravortty, D., Hensel, M., 2003. Inducible nitric oxide synthase and control of intracellular bacterial pathogens. Microbes Infect. 5, 621–627.

Defesche, J.C., 2004. Low-density lipoprotein receptor—its structure, function, and mutations. Semin. Vasc. Med. 4, 5–11.

Delneste, Y., 2004. Scavenger receptors and heat-shock protein-mediated antigen cross-presentation. Biochem. Soc. Trans. 32, 633–635.

De Sanctis, J.B., Blanca, I., Rivera, H., Bianco, N.E., 1998. Expression of low-density lipoprotein receptors in peripheral blood and tonsil B lymphocytes. Clin. Exp. Immunol. 113, 206–212.

Dunne, D.W., Resnick, D., Greenberg, J., Krieger, M., Joiner, K.A., 1994. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc. Natl. Acad. Sci. U.S.A. 91, 1863–1867.

Eckhardt, E.R., Cai, L., Sun, B., Webb, N.R., van der Westhuyzen, D.R., 2004. High density lipoprotein uptake by scavenger receptor SR-BII. J. Biol. Chem. 279, 14372–14381.

Gillotte-Taylor, K., Boullier, A., Witztum, J.L., Steinberg, D., Quehenberger, O., 2001. Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. J. Lipid Res. 42, 1474–1482.

Green, L., Wagner, D., Glogowski, J., Skipper, P., Wishnok, J., Tannenbaum, S., 1982. Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Gu, X., Kozarsky, K., Krieger, M., 2000. Scavenger receptor class B, type I-mediated [3H]cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. J. Biol. Chem. 275, 29993–30001.

Hampton, R.Y., Golenbock, D.T., Penman, M., Krieger, M., Raetz, C.R., 1991. Recognition and plasma clearance of endotoxin by scavenger receptors. Nature 352, 342–344.

Harvey, B.P., Quan, T.E., Rudenga, B.J., Roman, R.M., Craft, J., Mamula, M.J., 2008. Editing antigen presentation: antigen transfer between human B lymphocytes and macrophages mediated by class A scavenger receptors. J. Immunol. 181, 4043–4051.

He, H., Genovese, K.J., Lowry, V.K., Nisbet, D.J., Kogut, M.H., 2006a. Response of nitric oxide production to CpG oligodeoxynucleotides in turkey and chicken peripheral blood monocytes. FEMS Immunol. Med. Microbiol. 48, 99–106.

He, H., Genovese, K.J., Nisbet, D.J., Kogut, M.H., 2006b. Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes. Mol. Immunol. 43, 783–789.

He, H., Genovese, K.J., Nisbet, D.J., Kogut, M.H., 2007. Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. Mol. Immunol. 44, 3234–3242.

He, H., Kogut, M.H., 2003. CpG-ODN-induced nitric oxide production is mediated through Clathrin-dependent endocytosis, endosomal maturation, and activation of PKC, MEK1/2 and p38 MAPK, and NF-κB pathways in avian macrophage cells (HD11). Cell. Signal. 15, 911–917.

He, H., Lowry, V.K., Swaggerty, C.L., Ferro, P.J., Kogut, M.H., 2005. In vitro activation of chicken leukocytes and in vivo protection against *Salmonella enteritidis* organ invasion and peritoneal *S. enteritidis* infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide. FEMS Immunol. Med. Microbiol. 43, 81–89.

Holvoet, P., 1999. Endothelial dysfunction, oxidation of low-density lipoprotein, and cardiovascular disease. Ther. Apher. 3, 287–293.

Hsu, H.Y., Chiu, S.L., Wen, M.H., Chen, K.Y., Hua, K.F., 2001. Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. J. Biol. Chem. 276, 28719–28730.

Kogut, M.H., McGruder, E.D., Hargis, B.M., Corrier, D.E., DeLoach, J.R., 1995. *In vivo* activation of heterophil function in chickens following injection with *Salmonella enteritidis*-immune lymphokines. J. Leukoc. Biol. 57, 56–62.

Lara, L.L., Rivera, H., Perez-P, C., Blanca, I., Bianco, N.E., De Sanctis, J.B., 1997. Low density lipoprotein receptor expression and function in human polymorphonuclear leucocytes. Clin. Exp. Immunol. 107, 205–212.

Limmon, G.V., Arredouani, M., McCann, K.L., Corn Minor, R.A., Kobzik, L., Imani, F., 2008. Scavenger receptor class-A is a novel cell surface receptor for double-stranded RNA. FASEB J. 22, 159–167.

Matsuno, R., Aramaki, Y., Arima, H., Tsuchiya, S., 1997. Scavenger receptors may regulate nitric oxide production from macrophages stimulated by LPS. Biochem. Biophys. Res. Commun. 237, 601–605.

Mineo, C., Shaul, P.W., 2003. HDL stimulation of endothelial nitric oxide synthase: a novel mechanism of HDL action. Trends Cardiovasc. Med. 13, 226–231.

Mollenhauer, J., Wiemann, S., Scheurlen, W., Korn, B., Hayashi, Y., Wilgenbus, K.K., von Deimling, A., Poustka, A., 1997. DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. Nat. Genet. 17, 32–39.

Mukhopadhyay, S., Chen, Y., Sankala, M., Peiser, L., Pikkarainen, T., Kraal, G., Tryggvason, K., Gordon, S., 2006. MARCO, an innate activation marker of macrophages, is a class A scavenger receptor for *Neisseria meningitidis*. Eur. J. Immunol. 36, 940–949.

Mytar, B., Gawlicka, M., Szatanek, R., Wołoszyn, M., Ruggiero, I., Piekarska, B., Zembala, M., 2004. Induction of intracellular cytokine production in human monocytes/macrophages stimulated with ligands of pattern recognition receptors. Inflamm. Res. 53, 100–106.

Nakamura, T., Suzuki, H., Wada, Y., Kodama, T., Doi, T., 2006. Fucoidan induces nitric oxide production via p38 mitogen-activated protein kinase and NF-kappaB-dependent signaling pathways through macrophage scavenger receptors. Biochem. Biophys. Res. Commun. 343, 286–294.

Peiser, L., De Winther, M.P., Makepeace, K., Hollinshead, M., Coull, P., Plested, J., Kodama, T., Moxon, E.R., Gordon, S., 2002a. The class A macrophage scavenger receptor is a major pattern recognition receptor for *Neisseria meningitidis* which

is independent of lipopolysaccharide and not required for secretory responses. Infect. Immun. 70, 5346–5354.
Peiser, L., Mukhopadhyay, S., Gordon, S., 2002b. Scavenger receptors in innate immunity. Curr. Opin. Immunol. 14, 123–128.
Plüddemann, A., Mukhopadhyay, S., Gordon, S., 2006. The interaction of macrophage receptors with bacterial ligands. Expert Rev. Mol. Med. 8, 1–25.
Plüddemann, A., Neyen, C., Gordon, S., 2007. Macrophage scavenger receptors and host-derived ligands. Methods 43, 207–217.
Rosenstiel, P., Sina, C., End, C., Renner, M., Lyer, S., Till, A., Hellmig, S., Nikolaus, S., Fölsch, U.R., Helmke, B., Autschbach, F., Schirmacher, P., Kioschis, P., Hafner, M., Poustka, A., Mollenhauer, J., Schreiber, S., 2007. Regulation of DMBT1 via NOD2 and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. J. Immunol. 178, 8203–8211.
Shakushiro, K., Yamasaki, Y., Nishikawa, M., Takakura, Y., 2004. Efficient scavenger receptor-mediated uptake and cross-presentation of negatively charged soluble antigens by dendritic cells. Immunology 112, 211–218.
Thomas, C.A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S.C., El Khoury, J., 2000. Protection from lethal Gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. J. Exp. Med. 191, 147–156.
Trigatti, B., Covey, S., Rizvi, A., 2004. Scavenger receptor class B type I in high-density lipoprotein metabolism, atherosclerosis and heart disease: lessons from gene-targeted mice. Biochem. Soc. Trans. 32, 116–120.
Vandaveer, S.S., Erf, G.F., Durdik, J.M., 2001. Avian T helper one/two immune response balance can be shifted toward inflammation by antigen delivery to scavenger receptors. Poult. Sci. 80, 172–181.
van der Laan, L.J., Döpp, E.A., Haworth, R., Pikkarainen, T., Kangas, M., Elomaa, O., Dijkstra, C.D., Gordon, S., Tryggvason, K., Kraal, G., 1999. Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. J. Immunol. 162, 939–947.
Villwock, A., Schmitt, C., Schielke, S., Frosch, M., Kurzai, O., 2008. Recognition via the class A scavenger receptor modulates cytokine secretion by human dendritic cells after contact with *Neisseria meningitidis*. Microbes Infect. 10, 1158–1165.